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Repair of the tRNA-Like CCA Sequence in a Multipartite Positive-Strand RNA Virus

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The 3′ portions of plus-strand brome mosaic virus (BMV) RNAs mimic cellular tRNAs. Nucleotide substitutions or deletions in the 3′ CCA of the tRNA-like sequence (TLS) affect minus-strand initiation unless repaired. We observed that 2-nucleotide deletions involving the CCA 3′ sequence in one or all BMV RNAs still allowed RNA accumulation in barley protoplasts at significant levels. Alterations of CCA to GGA in only BMV RNA3 also allowed RNA accumulation at wild-type levels. However, substitutions in all three BMV RNAs severely reduced RNA accumulation, demonstrating that substitutions have different repair requirements than do small deletions. Furthermore, wild-type BMV RNA1 was required for the repair and replication of RNAs with nucleotide substitutions. Results from sequencing of progeny viral RNA from mutant input RNAs demonstrated that RNA1 did not contribute its sequence to the mutant RNAs. Instead, the repaired ends were heterogeneous, with one-third having a restored CCA and others having sequences with the only commonality being the restoration of one cytidylate. The role of BMV RNA1 in increased repair was examined.

The 3′ ends of viral RNAs are required for the proper translation, stability, and replication of the RNAs. For RNA replication, the end of the RNA must be unwound and recognized by the viral replication machinery (7). Consistent with this need, several RNA-dependent RNA polymerases (RdRps) have a narrow template channel that can only accommodate single-stranded RNA or have mechanisms that discriminate against the use of double-stranded templates for de novo initiation (10, 43, 53). A potential cost of having a single-stranded 3′ sequence is increased susceptibility to cellular nucleases. It is therefore to be expected that RNA viruses have mechanisms to protect the ends from degradation and/or to repair the end sequences.

Strategies that could prevent degradation include the formation of base paired structures that can be opened through alternative base pairing, as in carmovirus (51), the binding of cellular proteins, as in phage Qβ (5), or covalent linkage of viral proteins to the ends of viral genomes, as in picornaviruses and some DNA viruses (60). Several viral RNA repair processes have also been identified. The 3′ ends of viral RNA genomes and their associated satellite (sat) RNAs can be repaired either by viral polymerase, by RNA recombination, or by a host-terminal transferase, including the poly(A) polymerase complex (9, 11, 12, 19, 20, 28, 31, 47, 48, 61). Minus-strand viruses such as *Hantaan virus* (29) and *Respiratory Syncytial virus* (41) have ends with short repeats that apparently allow initiation of RNA synthesis within an internal repeat and then realign the nascent RNA at the end of the genome, thereby regenerating the ends of the RNAs. Other strategies may include the use of abortive initiation products or the synthesis of initiation products from mutated initiation sequence to prime the synthesis of the turnip crinkle virus (TCV) satellite RNA (10, 47).

A number of plant-infecting RNA viruses have structures that resemble cellular tRNAs with the hallmark CCA motif (15, 24). These tRNA-like sequences (TLS) are multifunctional. They increase the stability of the RNA, direct initiation of minus-strand RNA synthesis, promote translation, and contribute to increased repair by recruiting cellular enzymes that repair damaged cellular tRNAs (4, 24, 38, 54). In comparison to that of tombusviruses and RNA viruses with 3′ poly(A) tails, however, our understanding of the end repair of the viral TLS is quite limited. An examination of the repair of viral TLS could reveal interactions between viral and cellular processes.

Brome mosaic virus (BMV), the type member of the genus *Bromovirus*, is a tripartite positive-strand RNA virus where all three genomic and the subgenomic RNAs have nearly identical 3′ TLSs with the canonical CCA sequence at their ends (27, 36). RNA1 and RNA2 encode replication proteins named 1a and 2a, respectively. 1a and 2a associate with host factors to form a membrane-associated replicase (62) (for a review of host factors associated with viral replication, see references 2 and 42). RNA3 encodes the 3a movement protein and the capsid protein that is translated from subgenomic RNA4 (37). The 3′ TLS of the three BMV RNAs presents several opportunities for RNA end repair, such as RNA recombination (8, 58), the use of abortive products to prime RNA synthesis, and repair by cellular (ATP, CTP):tRNA nucleotidyltransferase (22, 54).

In this report, we found that the repair of nucleotide substitutions and deletions of the CCA sequence in BMV RNAs have different requirements. Further, wild-type RNA1 was necessary for the repair of the other two BMV RNAs with nucleotide substitutions in their CCA ends. Potential roles of viral and cellular enzymes in the repair of the BMV TLS are discussed.

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MATERIALS AND METHODS

Nomenclature. The number of different mutant RNAs examined in this study and the use of combinations of RNAs with wild-type and mutant ends require an intuitive nomenclature. For brevity, we will refer to BMV RNAs as R1, R2, and

R3. In general, an RNA with a mutation in the CCA site will have the mutation as part of its name. As examples, R1 with a change of CCA to GGA will be named R1GGA, and R2 with a deletion of the 3′-terminal two nucleotides, CA 3′, will be named R2Δ2 (Fig. 1A). Additional mutant RNA names will be explained as necessary.

RNA synthesis in vitro. DNA templates for in vitro transcription were generated by use of the plasmids described by Janda et al. (35), Vent polymerase (New England Biolabs), and pairs of oligonucleotide DNA primers (see Table 1 for sequences of oligonucleotides). The sense primers to generate cDNAs for R1 to R3 synthesis were named B0.1, B0.2, and B0.3 and contained a promoter for the T7 RNA polymerase. The antisense primer named B1-3wt was used for the 3′ end of the cDNA. GGA substitutions were made with the antisense primer B1-3GGA, and deletions of 2, 4, 5, 6, and 8 nucleotides were made with antisense primers B1-3Δ2, B1-3Δ4, B1-3Δ5, B1-3Δ6, and B1-3Δ8, respectively. To mark the 3′-terminal sequence of R1, the fourth nucleotide from the 3′ end was altered from a U to an A by use of antisense primer B1M4. To mark the fourth nucleotide of R2GGA and/or R3GGA, antisense primer B2,3M4 was used.

Capped full-length transcripts were made by use of the Amplicap T7 High Yield Message Maker kit as described by the manufacturer (Epicentre Inc., Madison, Wis.). Transcripts were precipitated with $LiCl_2$ to remove free nucleotides, visually analyzed by agarose gel electrophoresis followed by Toluidine blue staining, and quantified by spectroscopy.

Construction of chimeric BMV RNAs. Chimeras with precise replacements of the 5′-untranslated regions (UTRs) and protein-coding sequences were made by use of a combination of recombinant PCR, site-directed mutagenesis, and swapping of restriction fragments into the appropriate backbone, according to the protocol of Higuchi et al. (34). DNA sequencing confirmed that all cDNAs had only the intended changes. Due to the complex nature in which these constructs were made, details and maps will be provided upon request.

Protoplast inoculations and analysis of progeny viral RNA. Protoplasts were isolated from 5-day-old barley (*Hordeum vulgare* cv. Apex) primary leaves as described by Hema and Kao (33) and based on the protocols of Kroner et al. (40). Briefly, 10^6 cells were transfected with 0.5 μg of each combination of capped transcripts. Total RNAs were extracted from transfected protoplasts by using a lysis buffer (100 mM glycine, 10 mM EDTA, 100 mM NaCl, 2% sodium dodecyl sulfate, and 0.05% bentonite), usually at 12 h posttransfection unless stated otherwise. The RNA was quantified by spectrophotometry, denatured with glyoxal, and subjected to gel electrophoresis. Total RNAs were transferred onto nylon membranes (Nytran; Schleicher & Schuell), and the images of ribosomal RNAs (rRNAs) were photographed. The blots were probed using strand-specific riboprobes that detect the 3′ 200 nucleotides of the BMV RNAs. Each blot was probed to sequentially detect minus- and plus-strand RNAs, stripping off the bound probe in a low-salt buffer at 95°C between analyses. Time course experiments were performed with a set of independently transfected protoplasts, pooled after transfection to normalize for possible differences in transfection efficiency, and aliquoted for incubation. Total RNAs were harvested at the indicated times and were stored at −70°C until the entire set of samples was ready for further processing.

All quantifications were performed with a PhosphorImager and Image Quant programs. Results from mutant RNAs were normalized to the wild-type BMV transcripts in the same experiment. Two additional and independent sets of transfections in a subsequent experiment(s) was performed to ensure the consistency of the results.

Sequencing of viral cDNAs. Protoplasts transfected with desired transcripts for 48 h were used to enrich for BMV virions by centrifugation through a 10% sucrose cushion as described by Rao et al. (56). Viral RNAs were extracted from purified virions as described previously (32). These RNAs were subjected to polyadenylation with poly(A) polymerase and 5 mM rATP for 30 min at 37°C, and then they were subjected to cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Inc.) with an oligo$(dT)_{18}$ primer at 37°C for 60 min. PCR amplification of the cDNA used the oligo$(dT)_{18}$ primer and a BMV RNA-specific sense primer. We found that reverse transcription-PCR (RT-PCR) fragments derived from BMV RNAs that had wild-type CCA ends could be sequenced directly. However, heterogeneity in repaired products from RNAs with mutated CCA sites required the elution of the RT-PCR fragments and cloning into pGEM-TEasy (Promega Inc.) prior to sequencing of individual clones.

RESULTS

Requirements for the repair of deletions and nucleotide substitutions. We observed previously that a single-nucleotide

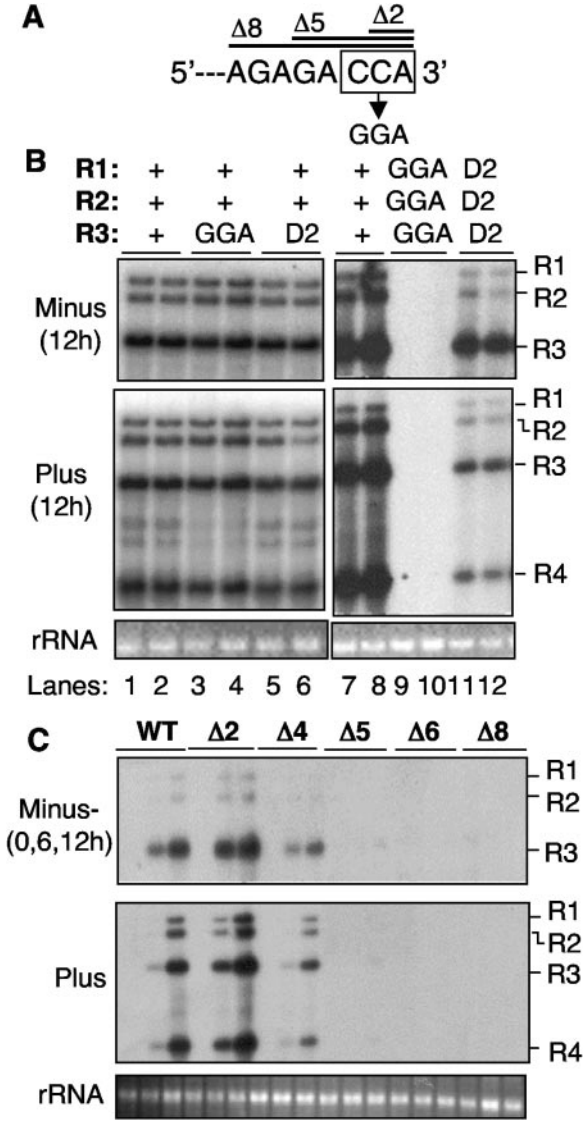


FIG. 1. One or more BMV RNAs are required for the repair of nucleotide substitutions but not for small deletions. (A) Sequence of the 3′ end of all BMV positive-strand RNAs and the mutations that affect the CCA site. The horizontal bars denote the nucleotide deleted, with the names of the deletion written above the bar. The box identifies the CCA sequence, which was changed to GGA when nucleotide substitutions were studied. (B) Effects of mutations in one or more BMV RNAs. The gel images were all taken from one Northern blot experiment by using the Molecular Dynamics program Data Storm. The sense of the RNAs is denoted to the left, and the time at which the protoplasts were harvested is denoted in parentheses (12 h in this experiment). The identities of individual RNAs are listed to the left and right of the images. The barley protoplasts were transfected with a mixture of three RNAs, either wild type (WT) (denoted by a plus sign) or with a mutation. The two slices of gel near the bottom are the 18S rRNA stained with ethidium bromide. In general, all of the gel images in this work are arranged in the same format. (C) Effect of short deletions in one or more BMV RNA repairs and replications. In this experiment, all three of the BMV RNAs had the deletion from the 3′ end. The names of the mutants indicate the number of nucleotides deleted. Protoplasts were transfected and then harvested at 0, 6, and 12 h posttransfection, as labeled below the gel image. The identities of the RNA bands are indicated to the side of the gel images.

TABLE 1. Names and sequences of DNA primers used in this study

Name	Sequence (5′ to 3′)[a]
B.1	TAATACGACTCACTATAGTAAACCACGGAACGAGGTTCAATC
B.2	TAATACGACTCACTATAGTAGACCACGGAACGAGGTTCAATC
B.3	TAATACGACTCACTATAGTAAAATACCAACTAATTCTCGTTC
B1-3wt	TGGTCTCTTTTAGAGATTTACAGTGTT
B1-3GGA	TCCTCTCTTTTAGAGATTTACAGTGTT
B1-3Δ2	GTCTCTTTTAGAGATTTACAGTGTTTTTC
B1-3Δ4	CTCTTTTAGAGATTTACAGTGTTTTTCA
B1-3Δ5	TCTTTTAGAGATTTACAGTGTTTTTCA
B1-3Δ6	CTTTTAGAGATTTACAGTGTTTTTCA
B1-3Δ8	TTTAGAGATTTACAGTGTTTTTCA
B1M4	TGG**A**CTCTTTTAGAGATTTACAGTGTTT
B2,3M4	TCC**A**CTCTTTTAGAGATTTACAGTGTTT
dT_{18}	TTTTTTTTTTTTTTTTTT
B1RTPCR	GCGGCCCTTCAAACGAGGGCTAAGGATTTTC
B2RTPCR	GCATCAGAGTTTATCAGATGAGCGATCCTGT
B3RTPCR	CCTTGGCGGTTGCAGACTCCTCGAAAGAGG

[a] Underlining denotes primers that contained a promoter for the T7 RNA polymerase; bold type and underlining denote mutations.

substitution or a 2-nucleotide deletion at the 3′ CCA end of R3 resulted in significant levels of R3 replication (33). We wanted to determine whether wild-type R1 and/or R2 contributed to the repair of the mutant R3. Therefore, barley protoplasts were transfected with combinations of BMV transcripts where one or more RNAs had the GGA substitution or a 2-nucleotide deletion of the CCA 3′ sequence (Fig. 1A). Consistent with our previous observations (33), R3GGA and R3Δ2 in the presence of wild-type R1 and R2 accumulated minus- and plus-strand RNAs at levels greater than 70% of that of wild-type R3 (Fig. 1B and Table 2). These results were observed at 12 h posttransfection, before maximal replication (usually at ~16 to 20 h), suggesting that the RNAs were rapidly repaired. BMV RNAs with deletion in all three 3′ ends also accumulated the genomic RNAs to at least 25% of wild-type transfection (Fig. 1B, lanes 11 to 12; Table 2). In contrast, protoplasts transfected with all three RNAs with GGA substitutions did not accumulate above background levels (Fig. 1B, lanes 9 to 10). These results indicate that BMV RNAs with short deletions are repaired differently in barley protoplasts than BMV RNAs with nucleotide substitutions.

To examine the repair of short deletions further, we deleted up to 8 nucleotides of the 3′ end of all three BMV RNAs and assessed whether the viral RNA syntheses could recover. RNAs with deletion of 4 nucleotides (5′ACCA3′) accumulated at a reduced level compared to that of a 2-nucleotide deletion, and deletions of 5 or more nucleotides were at background levels (Fig. 1C). The rest of this analysis will focus on nucleotide substitutions where there is a requirement for one or more wild-type BMV RNA. For brevity, we shall refer to the phenomenon of RNA repair followed by RNA replication as increased repair. In addition, because minus-strand RNA synthesis will be the first step affected by the mutations, we will present only the Northern blots detecting minus-strand RNAs unless there is a need to show an effect on subgenomic RNA4 accumulation. Plus-strand RNAs were detected for all key experiments shown in this work, and quantifications of the results are shown in Table 2. In general, the effects on plus-strand RNAs mirrored those of minus-strand RNA. An exception is that some combinations with chimeric R3/1 had differential reduction of plus-strand RNA accumulation (data not shown). This indicates differences in the requirements for replication, not repair, and was not pursued in this work.

Wild-type BMV R1 is required for increased repair of substitutions at the 3′ ends of R2 and R3. To determine which BMV RNA contributed to increased repair of 3′ ends of BMV RNAs, combinations of wild-type and mutated transcripts were transfected into barley protoplasts (Fig. 2A). Wild-type R1 in combination with R2GGA and/or R3GGA had increased repair. Minus-strand progeny from R3GGA accumulated to 123% of wild type, while those from R2GGA were at 38% of R2 (Fig. 2A, compare R2 bands in lanes 5 and 6 to those in lanes 1 and 2). Therefore, R3GGA is preferred over R2GGA as a repair substrate. To confirm and extend the result that R1 is required for increased repair, we examined BMV RNA synthesis at 0, 6, and 12 h after transfection (Fig. 2B). Wild-type R2 and/or R3 could not increase repair of any of the RNAs with GGA ends in the presence of wild-type R1, indicating a special role for R1 in this process (Fig. 2B, lanes 7 to 9 and 17 to 19; Table 2). This observation does not rule out, however, the possible involvement of the gene products from R2 and R3 in increased repair. Lastly, notable increases in RNA levels were observed even at 6 h after transfection, timing that is incompatible with RNA recombination being a major mechanism for repair, because recombination is likely to be at low frequency (54, 70). Again, we observed more rapid repair of R3GGA than R2GGA (Fig. 2B).

Sequencing of progeny viral RNAs. We have two hypotheses as to how R1 contributes to increased repair. First, it may allow BMV RNAs to exist in a state that could be repaired, perhaps by stabilizing BMV RNAs and/or localizing them to specialized sites, as has been proposed for BMV replication in yeast (16, 17, 60). Second, it may contribute its nucleotide sequence to the mutated RNA, perhaps through a high rate of replicase ternary complex initiating synthesis from R1, translocating to R2 and/or R3, and completing RNA synthesis. Abortive synthesis from the 3′ end of BMV RNAs was characterized in vitro (67), and there is precedent for their participation in the repair of TCV satellite RNAs (12, 47). We sequenced the ends of the putatively repaired RNAs to distinguish between these two possibilities.

The last few nucleotides of all three BMV RNAs are normally identical, making it difficult to track whether the R1 sequence was transferred to the repaired RNAs. Therefore, we marked R1 with a base substitution at the −4 position from the 3′ end, changing the wild-type A to a U (5′G**U**CCA3′; the marked U is underlined) in an RNA named $R1^m$. This change should not affect the formation of the TLS (25), and it did not interfere with the increased repair of R2GGA and R3GGA (Fig. 3A). Encapsidated progeny RNAs generated from this input mixture were sequenced. All cDNAs generated from the

TABLE 2. Summary of the RNAs accumulated in the repair experiments shown in this study

Construct(s) and figure in which they appear[a]	Minus strand (percentile)			Plus strand (percentile)			
	R1	R2	R3	R1	R2	R3	R4
R1, R2, R3	100	100	100	100	100	100	100
Fig. 1B							
R1, R2, **R3GGA**	159	158	89	128	113	93	82
R1, R2, **R3Δ2**	91	95	86	71	53	69	73
R1GGA, **R2GGA**, **R3GGA**	0	0	0	0	0	0	0
R1Δ2, **R2Δ2**, **R3Δ2**	42	36	59	39	27	30	21
Fig. 2A and B (12-h sample only)							
R1GGA, **R2GGA**, **R3 GGA**	0	0	0	0	0	0	0
R1, **R2GGA**, **R3GGA**	135	38	123	175	43	65	42
R1GGA, R2, **R3GGA**	0	0	0	0	0	0	0
R1, R2, **R3GGA**	220	195	98	152	124	76	70
Fig. 4B							
R1SLC^m, R2, R3	0	0	0	0	0	0	0
R1SLC^m, R2, **R3GGA**	0	0	0	0	0	0	0
R1SLC^m, **R2GGA**, **R3GGA**	0	0	0	0	0	0	0
Fig. 5C							
R1GGA, **R2GGA**, **R3GGA**	0	15	20	0	0	5	12
R2/1/2, **R2GGA**, R3	7	18	35	2	0	38	27
R2/1/2, R2, **R3GGA**	59	110	159	72	62	144	154
R2/1/2, **R2GGA**, **R3GGA**	4	10	67	35	29	36	43
Fig. 5D							
R2/1/2, R1, R2, R3	60	31	70	67	43	84	71
R2/1/2, **R2GGA**, **R3GGA**, **R1SLC^m**	129	58	130	107	86	151	135
Fig. 6A							
R2/1/2, **PK6**, **R2GGA**, **R3GGA**	33	20	39	7	10	39	30
R2/1/2, **PK6**, R2, **R3GGA**	80	70	39	53	74	96	64
Fig. 6B							
R2/1/2, **PK17**, **R2GGA**, **R3GGA**	17	9	21	23	11	62	31
R2/1/2, **PK17**, R2, **R3GGA**	28	20	33	53	68	94	58

[a] Mutated RNAs are in bold. Each value represents results at least two independent transfections taken at 12 h posttransfection. All results were normalized to the wild-type RNAs tested within the same experiment.

input R2GGA and R3GGA had at least one cytidylate near the poly(A) tail added during the analysis (Fig. 3D). This was expected, because only one cytidylate at the second or third position from the 3′ end of the RNA could direct minus-strand RNA synthesis (14, 67).

There were two interesting observations. First, none of the 13 progeny RNAs from R2GGA or R3GGA had a U at the fourth position, suggesting that R1 did not produce oligonucleotide primers that were used in repair. Second, only 4 of the 13 progenies regenerated a CCA sequence. The CCA enzyme is highly specific (61, 63), indicating that the tRNA nucleotidyltransferase was not solely responsible for the repaired sequences. To confirm the DNA sequencing results described above, we marked R2 and R3 by changing their −4As to Us. When in possession of a GGA substitution, these RNAs were repaired when R1 was present (Fig. 3A, lanes 4 to 5). Fifteen progeny RNAs from two independent transfections yielded sequencing results consistent with those from R1^m above (Fig. 3C). Altogether, repair to form a wild-type CCA was only seen in 9 of 28 progenies. The other cDNAs had haphazardly repaired sequences, with the only theme being the restoration of a cytidylate near the 3′ end. We therefore conclude that the majority of increased repair was not due to R1 contributing its sequence. The remainder of this work attempts to elucidate how R1 participated in repair.

Effects of R1 replication and concentration on increased repair. To examine whether increased repair requires specific amounts of input R1, it was transfected into protoplasts at 0 to 1 μg along with constant amounts (0.5 μg) of R2GGA and R3GGA. Increased repair of R2GGA and R3GGA was correlated with the amount of R1 in the initial transfection; maximum repair required approximately 0.5 μg of R1 per transfection (Fig. 4A and B).

We next determined whether a mutation that eliminated R1 minus-strand RNA synthesis would affect repair. Stem loop C (SLC) within the TLS has a terminal hairpin that forms a clamped adenine motif, which binds the BMV replicase and directs minus-strand initiation (5′AUA3′; the key adenine is underlined) (Fig. 4B) (14, 37). Preventing the formation of a clamped adenine in R3 eliminated minus-strand RNA synthesis in vitro and in barley protoplasts (18, 39, 64). R1SLC^m, which had the clamped adenine changed to a guanine, was defective for both replication and increased repair of R2GGA and R3GGA (all three RNAs were transfected at 0.5 μg per

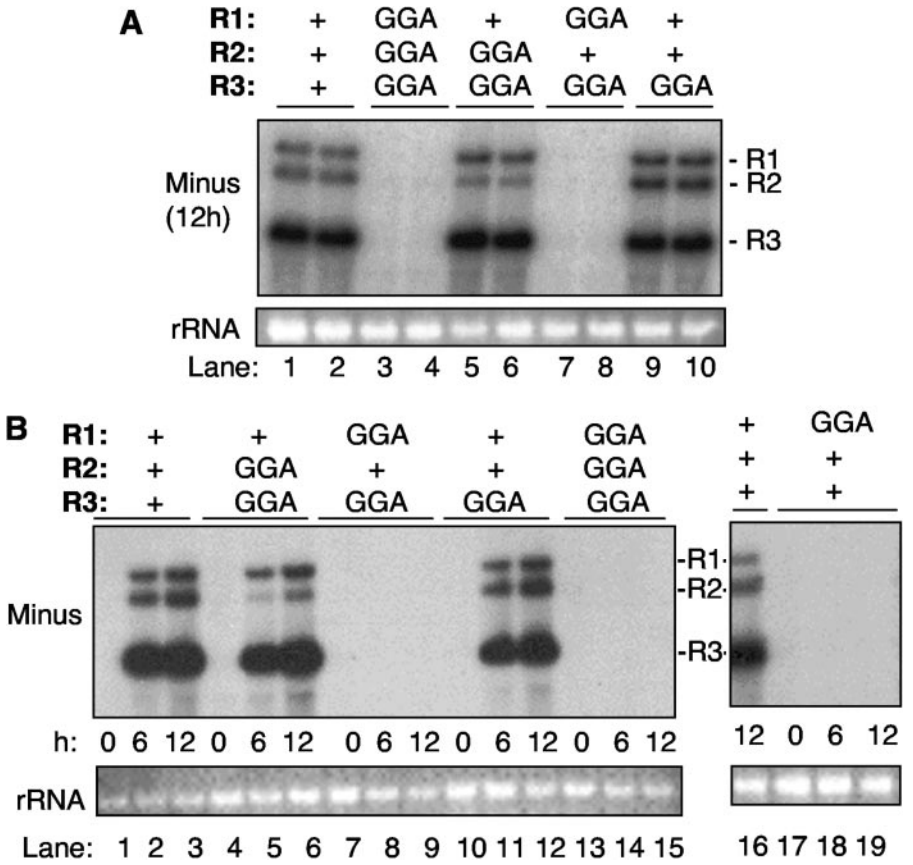


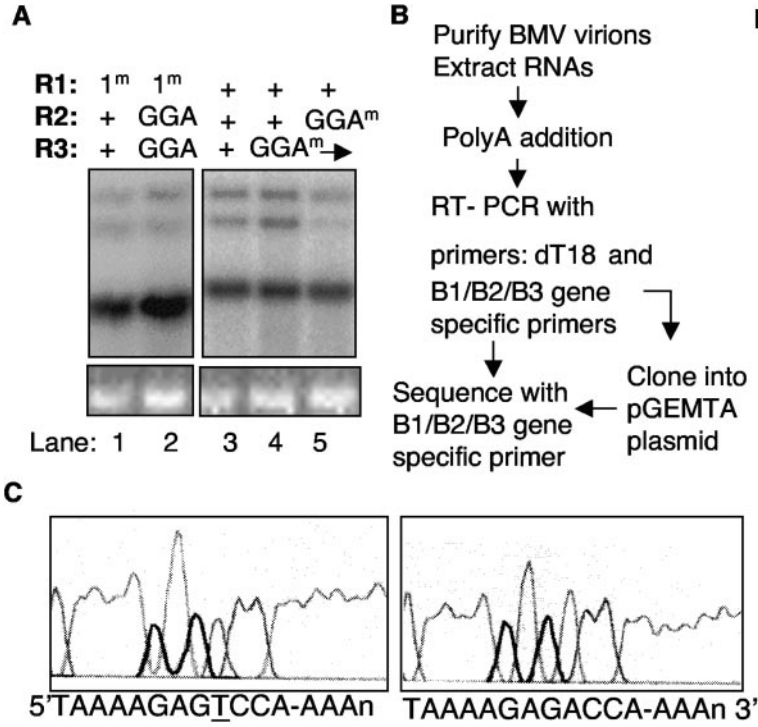
FIG. 2. R1 is required to increase the repair of base substitutions in R2 and R3. (A) Gel image of a Northern blot containing different combinations of wild-type and mutant RNAs, as denoted above the gel image. The polarity of the RNAs is shown to the left of the image, and the identity of each RNA is indicated to the right. Similar results were observed for the plus-strand RNAs (data not shown). (B) A time course experiment to confirm that R1 is required for increased repair of R2 and R3 with base substitutions at their 3′ ends and that R3 is a preferred substrate for repair in comparison to R2. The times, in hours, at which the transfected protoplasts were harvested are stated at the bottom of the gel image.

reaction) (Fig. 4C). This result and those from Fig. 4A indicate that the amount of R1 and/or 1a in transfected protoplasts is correlated with increased repair.

Examination of chimeric RNAs. We used chimeric RNAs to determine whether the 1a coding sequence or the R1 UTRs are required for increased repair. Chimeras of R2 and R3 with their 3′ TLS replaced with the sequence from R1 were unable to repair either R2GGA or R3GGA (data not shown). A key chimera in these studies is R2/1/2, which contains the 5′ and 3′ UTRs of R2 flanking the 1a coding sequence (Fig. 5A). R2/1/2 could replace R1 and direct wild-type levels of BMV RNAs (Fig. 5B). R2/1/2 was also able to direct increased repair of R3GGA (accumulating to 159% of R3) when R2 was wild type (Fig. 5C, lanes 7 and 8). However, in reactions where R2 required repair, R2/1/2 was only able to help the R2GGA progeny accumulate to 18% of R2 (Fig. 5C, lanes 5 and 6; Table 2). In reactions with both R2GGA and R3GGA, their progenies accumulated at 10 and 67%, respectively, of the wild-type RNAs (Fig. 5C, lanes 9 to 10; Table 2). This is higher than levels seen with R1GGA but lower than that of the repair of R2GGA and R3GGA by wild-type R1 (Table 2). These results suggest that R2/1/2 could produce amounts of 1a sufficient for the replication of RNAs without mutated initiation sites but could not fully replace R1 for increased repair.

To examine whether an additional source of 1a could function along with R2/1/2 to repair both R2GGA and R3GGA, we added the replication-defective R1SLC^{m} (with a defect in the clamped adenine; Fig. 4B) to transfections with R2/1/2, R2GGA, and R3GGA. In this manipulation, we are assuming that R1SLC^{m} could be translated to produce 1a. In agreement with the results of Rao et al. (55), we have observed that R2SLC^{m} could produce sufficient amounts of 2a for detectable levels of R1 and R3 replication (18). These transfected cells were able to accumulate minus strands of R2GGA and R3GGA at 58 and 130% of the respective wild-type RNAs, levels similar to those of reactions where R1 was present (Fig. 5D, compare lanes 7 and 8 to 1 and 2; Table 2). These results are consistent with the idea that increasing the abundance of the 1a protein could result in increased repair.

Effects of mutations in the 1a coding sequence. To correlate further that functional 1a is required for increased repair, we tested the effects of PK6 and PK17, two insertion mutations that made R1 defective for replication (40). In yeast, 1a derivatives from PK6 and PK17 accumulated, respectively, at 60 and



D

Input RNAs	cDNA(form)	Sequence	#
R1m	cR1(PCR)	GUCCA	2
R2AGGA	cR2(plasmids)	GACCA	4*
R3AGGA		GACAA	1
		GAGCA	2
	cR3(plasmids)	GACAA	4
		GAGCA	2
R1	cR1(PCR)	GACCA	2
R2UGGA	cR2(plasmids)	GUGCA	3
R3UGGA		GUGCU	2
	cR3(plasmids)	GUGCU	1
		GUGCG	2
		GUCCA	2*
R1	cR1(PCR)	GACCA	2
R2	cR2(PCR)	GACCA	2
R3UGGA	cR3(plasmids)	GUGCA	1
		GUCCA	3*
		GUGCG	1

*Conversion to CCA
Percent with conversion: 9 of 28 (32%)

FIG. 3. Sequencing analysis of repaired RNAs in the presence of wild-type R1. (A) Gel images that demonstrate that R1 with a marked fourth position from the 3′ end (a change from A to U) is capable of replication as well as increased repair and that similarly marked R2GGA and R3GGA are substrates for the repair process. (B) Protocol used to sequence RT-PCR fragments directly or to clone and sequence individual sequences of the 3′ ends of R2GGA and/or R3GGA. RNAs that contained a CCA site that did not require repair were sequenced directly from PCR products, while the products of the repair were heterogeneous and required cloning into pGEM-TEasy. (C) Examples of the sequencing results from the RT-PCR product from $R1^m$ (left-hand graph) and from RT-PCR of R2 (right-hand graph). These results demonstrate that there is predominantly only one sequence in each of the two reactions. (D) Compilation of the DNA sequences from three independent experiments where different RNA combinations were transfected into barley protoplasts. The input RNAs are listed to the left. The column headed cDNA (form) identifies the origin of the RNAs used to generate the cDNA and whether they were sequenced after PCR or after cloning into pGEM-TEasy. The sequences observed and the number of independent reactions in which a sequence was observed is in the last column. Wherever the −4 position was marked to distinguish an RNA from the others, the marked nucleotide is underlined. The retention of the mutated residues is denoted with a boldface G. The number of sequences that originated from a mutant RNA is in boldface. The asterisks identify sequences that were restored to CCA and could be due to the activity of the cellular tRNA nucleotidyltransferase. The frequency of this repair to CCA is summarized at the bottom of the table.

90% of the level of wild-type 1a, indicating that they were not grossly unstable (50). Both PK6 and PK17 were incapable of replication, as expected from the results of Kroner et al. (40). They were also incapable of increased repair of either R2GGA or R3GGA (Fig. 6A and B, lanes 3 to 8, upper and lower panels). These results, along with those from $R1SLC^m$ (Fig. 4B), indicate that the R1 sequence alone is incapable of increased repair. To confirm that 1a produced from R2/1/2 could rescue repair, we cotransfected it into barley protoplasts along with R2GGA, R3GGA, and either R1PK6 or R1PK17 and found that there were significant levels of increased repair (Fig. 6A and B, lanes 9 to 12).

DISCUSSION

The maintenance of functional ends of viral RNAs should be a priority for the fitness of the virus. RNA viruses with multipartite genomes that contain a 3′ TLS could theoretically be repaired by RNA-RNA recombination mediated by the viral replicase, by other replication-associated activities of the viral replicase, such as abortive synthesis coupled with priming, or by cellular and/or viral enzymes that can directly repair the RNA (6, 8, 47, 54, 57, 58). To examine the repair of the CCA sequence, we tested one or more BMV RNAs with short deletions or nucleotide substitutions and found that repair was not only rapid (occurring within 6 h after transfection) but also could allow BMV RNAs to accumulate to significant levels (Fig. 1C and 2B). The kinetics of repair rule out RNA recombination as a major contributor to the amount of replication-competent RNA, in agreement with the previous observations of Rao et al. (54) wherein repair was observed at 2.5 h after transfection. We also observed different levels of repair for R3 versus R2 and a need for wild-type R1 for the repair of nucleotide substitutions (but not short deletions). Lastly, we observed that the repaired sequence is heterogeneous, with the only common theme being the restoration of one cytidylate that may be needed for the initiation of minus-strand RNA synthesis. This study provides an initial survey of the functions within the BMV RNAs that are required for end repair and replication.

Are viral or cellular enzymes responsible for repair? Our study does not allow assignment of definitive roles for cellular

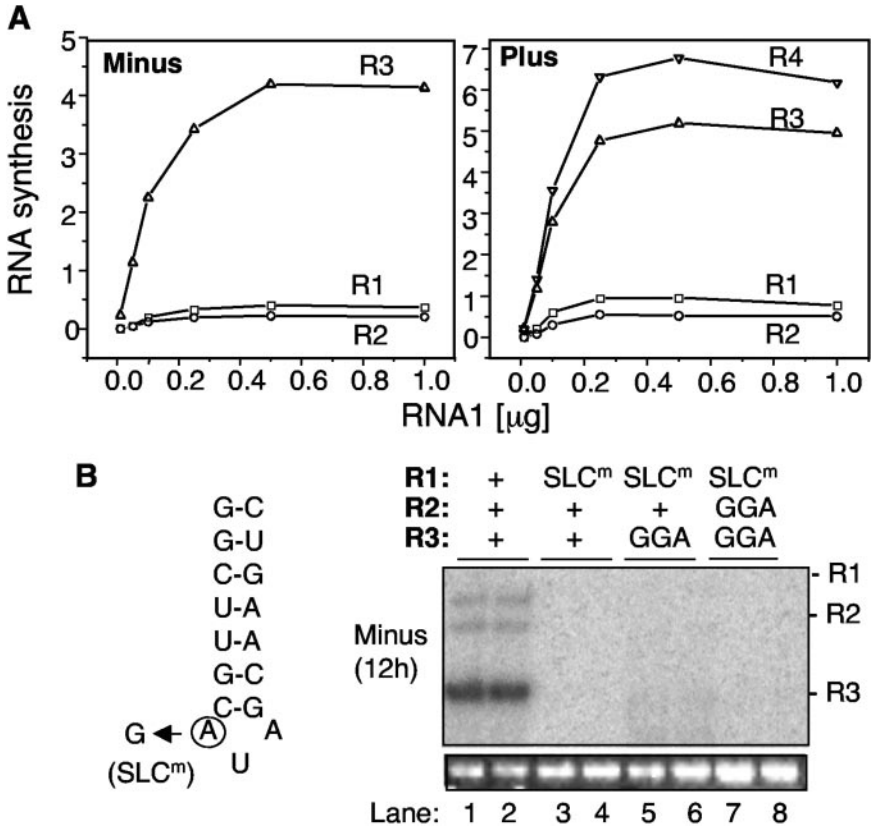


FIG. 4. Increased repair depends on R1 concentration and replication competence. (A) Effects of increasing amounts of R1 on the accumulation of different BMV RNAs. The RNAs transfected consisted of the amount of R1 indicated in the horizontal axis and 0.5 μg each of R2GGA and R3GGA. The amount of each class of RNA synthesized is represented in the vertical axis. (B) Mutations that affect minus-strand R1 synthesis prevented increased repair. The RNA stem-loop shown is the SLC within the tRNA-like region of all BMV plus-strand RNAs. The circled A is the clamped adenine that is required for minus-strand RNA initiation. A change to a G that would eliminate minus-strand RNA synthesis is identified by the name SLC^m. To the right of the schematic is the gel image from a Northern blot demonstrating that SLC^m in R1 eliminated both BMV replication and the repair of nucleotide substitutions. Wild-type RNAs are identified by a plus sign, while nucleotide substitutions in the CCA site of each RNA are identified by GGA.

or viral enzymes in end repair, primarily because we used the ability to replicate as a filter to identify the repaired RNAs. It is possible that the BMV replicase actively participates and/or is responsible for end repair. The 2a protein is needed only in catalytic amounts, hence even in cells transfected with R2GGA, sufficient amount of 2a could be produced to form the replicase (18, 55). The 1a protein is also needed in excess of 2a, consistent with our observation that wild-type R1 must be present and that increased amounts would increase repair (Fig. 4A).

The BMV replicase produced from transcripts with short deletions or nucleotide substitutions must be able to synthesize RNAs from these templates. Mutations in the CCA sequence of BMV RNAs have eliminated minus-strand initiation in vitro (15, 37, 67), suggesting that the BMV replicase cannot initiate RNA synthesis from the affected templates. However, two biochemical activities of the BMV replicase should be considered in evaluating whether the BMV replicase could reconstruct the affected CCA ends prior to RNA synthesis: nontemplated nucleotide addition and priming by aborted initiation products.

Nontemplated nucleotide addition is an activity common to DNA- and RNA-dependent RNA polymerases and can confer the ability to initiate from a short template that lacks an initiation cytidylate (31, 52, and references therein). While it could account for the heterogeneous end sequences we observed (Fig. 3), we note that the nucleotide substitutions need to be removed prior to nontemplated nucleotide addition, an activity not observed with the BMV replicase in vitro. We have also not observed the BMV replicase to initiate minus-strand RNA synthesis from templates with a 3′ GGA substitution (15), as was reported with the TCV RdRp (31).

Abortive initiation takes place with both DNA-dependent and RNA-dependent RNA polymerases in vitro (30, 67, 71). In the T7 RNA polymerase, it is part of a regulatory step that allows the polymerase to commit to the template for elongative RNA synthesis (65). Theoretically, abortive products from R1 could prime minus-strand BMV initiation from the other RNAs with nucleotide substitutions. This situation would not explain the efficient repair of all three RNAs with 3-nucleotide deletions (Fig. 1C). Also in disagreement with this model, we do not see exchanges of the marked fourth nucleotide in repair reactions that require R1 (Fig. 3D). It is unlikely that the marked R1 contributed a 2-nucleotide primer (5′pppGpG)

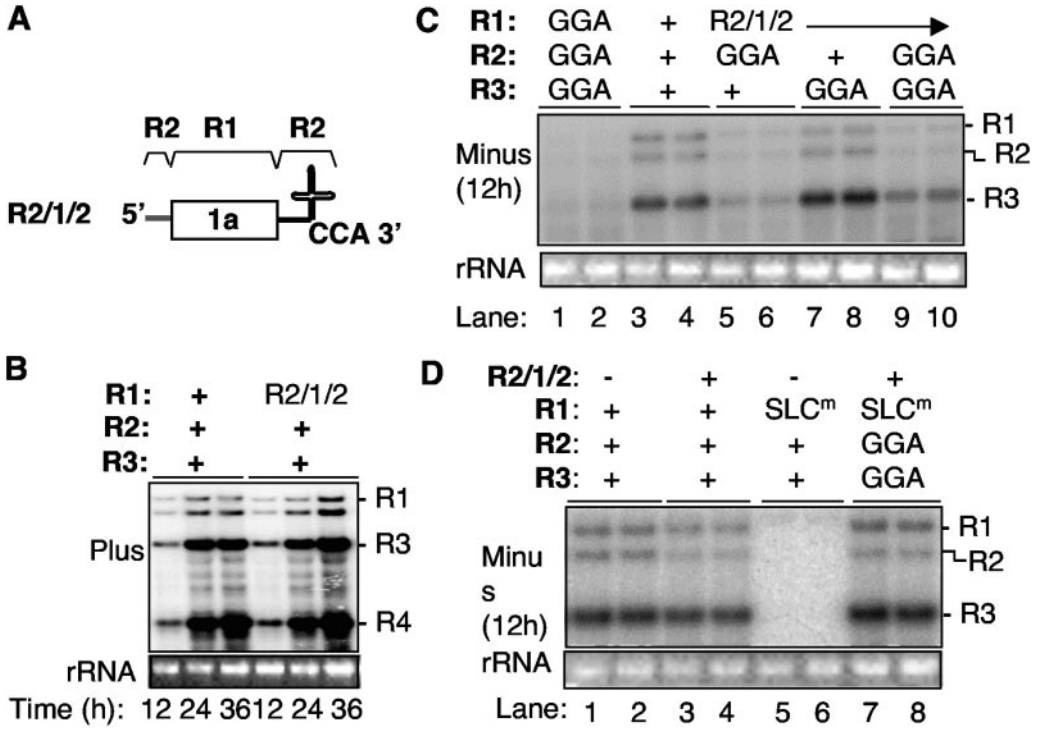


FIG. 5. Effects of a chimeric RNA on BMV replication and increased repair. (A) A schematic of chimeric RNA R2/1/2 used in this experiment. (B) Demonstration that R2/1/2 is capable of directing RNA replication and transcription. The levels of the RNAs were examined over a 2-day period to ensure that there was not a defect in RNA levels associated with R2/1/2 that is more obvious over time. (C) Examination of the ability of R2/1/2 to increase repair of R2GGA and R3GGA. The gel image is from a Northern blot of RNA harvested at 12 h posttransfection. The mixtures of the RNAs used in each transfection are identified above the gel image. (D) R2/1/2 and a replication-incompetent R1 can lead to the repair of R2GGA. Whether R2/1/2 or a wild-type RNA is present (+) or absent (−) is indicated. The presence of mutant versions of RNA is denoted by names instead of plus signs.

that participated in repair, because this dinucleotide could not base pair with the RNAs with GGA3′ ends. Position-dependent Watson-Crick base pairing is required for the use of initiation primers by the BMV replicase (37). Furthermore, many of the recovered sequences had only one cytidylate, not two (Fig. 3D). We interpret these results to mean that primer synthesis by the BMV replicase and subsequent extension is less relevant for the repair of the CCA ends of BMV RNAs.

Our observations of different requirements for the repair of short deletions and nucleotide substitutions (Fig. 1B) and the heterogeneity in the repaired sequence (Fig. 3D) suggest that the BMV replicase may not be solely responsible for end repair. One model is that both cellular and viral enzymes participate in reconstruction of the ends of the BMV TLS. Another is that the cellular enzymes would act first to reconstruct the CCA sequence or to generate a sequence that the BMV replicase could subsequently use to direct minus-strand initiation.

Should cellular proteins participate in BMV end repair, one likely candidate is the CCA tRNA nucleotidyltransferase that repairs deletions of the CCA ends of tRNAs (Fig. 1B) (22, 61). This enzyme has been demonstrated to bind to the BMV TLS sequence (27) and was postulated to repair the ends of viral RNAs, including those from BMV (54), the TLS of turnip yellow mosaic virus (44), and the yeast nanavirus (28). Consistent with a role of tRNA nucleotidyltransferase, increased repair was less efficient when the RNAs lacked more than four nucleotides at the 3′ end (ACCA3′) or when the CCA suffered substitutions (Fig. 1C). The tRNA nucleotidyltransferase recognizes only CTP and ATP as substrates and hence could not repair longer deletions that require other nucleotides (63, 73, 75). Furthermore, the tRNA nucleotidyltransferase lacks exonuclease activity, necessitating the removal of the substitutions other cellular enzymes prior to the reconstruction of the CCA sequence (3, 22).

In *Escherichia coli*, RNase T and/or polynucleotide phosphorylase (PNPase) can remove inappropriate nucleotides in the CCA sequence of tRNAs prior to end reconstruction by tRNA nucleotidyltransferase (13, 23, 75). Both PNPase and poly(A) polymerase can add heteropolymeric sequences to tRNAs similar to ones we observed in the repaired BMV RNAs (Fig. 3D) (3, 45, 76). PNPase has also been demonstrated to play a role in the maturation of plastid RNAs in *Arabidopsis* spp. (72). There is also a link between nonspecific cellular polymerase activities and viral RNA replication in the early days of RNA virology, prior to the demonstration that RNA viruses encode their own RdRps (38 and references therein). These disparate bits of results suggest that cellular polymerases would have an effect on the BMV TLS sequence early in the infection process.

The purported heavy reliance on cellular enzymes differs from previous observations from tombusviruses, where viral RNA repair is extensively studied. TCV and associated satRNAs with multiple virally mediated processes, including homologous and heterologous recombination, and abortive

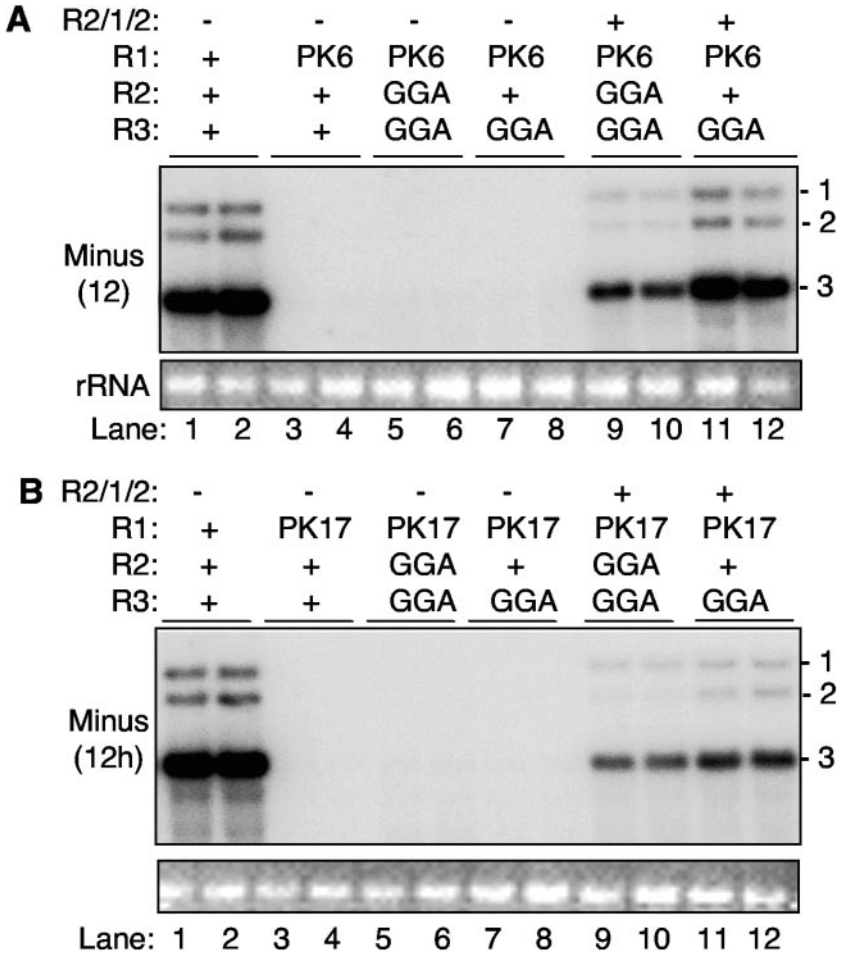


FIG. 6. Effects of mutations in the 1a coding sequence on increased repair. (A) Effect of replication-defective mutant PK6 on repair of R1GGA and R2GGA in the presence or absence of R2/1/2. A plus sign denotes the presence of RNA in the column. Specific names, such as PK6 and GGA, indicate that the RNA has a mutation. (B) Effects of replication-defective mutant PK17 on increased repair in the presence or absence of R2/1/2. The arrangement of the gel is the same as that for panel A. The identities of the RNAs are indicated to the sides of the gel images.

synthesis of 4 to 8 oligonucleotides coupled to use primers in the repair of satRNA ends and, potentially, base-independent initiation from any 3′-terminal sequence (11, 12, 31, 47). Members of the *Tombusviridae* have heteropolymeric 3′ ends but do not possess a TLS, perhaps necessitating more active participation of the viral replicase in repair. Another factor that may affect the mechanisms used for repair in BMV versus tombusviruses is that BMV does not possess natural satellite or defective interfering RNAs. Only one defective interfering RNA of BMV RNA3 could be generated after prolonged passage (21), indicating that either the BMV replicase might not leave a template RNA during RNA synthesis and reinitiate elsewhere as often as other viral replicases or that these BMV recombinants are selected against.

Role of R1 required in increased repair of nucleotide substitutions. BMV R1 contributed to increased repair of nucleotide substitutions at the CCA end. The 1a protein produced from R1 is likely the responsible component, because chimera R2/1/2, lacking the R1 UTRs, was capable of some level of increased repair (Fig. 5C). Replication-defective R1 with mutations in SLC or in-frame amino acid insertions in 1a at two different locations, PK6 and PK17, were unable to increase repair (Fig. 4B and 6), suggesting that the RNA sequence is not sufficient for increased repair. However, the situation is complex, because increased repair is a quantitative phenomenon that not only shows differences with repair of R2 and R3 but also can use the combined contributions of 1a from separate RNAs (Fig. 5D and 6). We have also tested two conditional mutations in the 1a protein for their effects on increased repair, PK19 and PK14. PK19 was reported to replicate well at 21°C but not at 34°C; PK14 replicates like the wild type at both temperatures (38). In our hands, they were both reduced for replication relative to R1, even at 21°C (data not shown). We do not understand the reason for this discrepancy from the results of Kroner et al. (40). However, both PK19 and PK14 were reduced in end repair at levels commensurate with those of RNA replication (data not shown). Again, this suggests that 1a function is tied to the level of repair. The repair of satellite RNAs associated with the tripartite *cucumber mosaic virus* also requires the presence of their RNA1 and RNA2 (9).

Several reports demonstrated that 1a has activities important for replication that are independent of its function in the

replicase complex. Ahlquist and colleagues have proposed that 1a stabilizes BMV RNA3 and RNA2 by binding to their respective B-box sequences and form specialized membrane-bound structures (2, 16, 17, 60, 66). Choi et al. (18) have demonstrated that the enriched BMV replicase, which contains 1a, does not bind the intercistronic B box. Whether 1a's role in increased repair is directly linked to the replicase needs to be addressed. Should 1a act independently of the replicase, it could stimulate the repair processes by altering the physiology inside the cell or by stabilizing BMV RNAs to increase the chance for repair. The latter interaction may be especially necessary for RNAs with nucleotide substitutions, because they need to be acted on by both nuclease(s) and repair polymerase(s). Should 1a binding to R3 and R2 have different affinities, this could explain the preferential repair of nucleotide substitutions in R3.

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